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14. ABSTRACT
Genetic breast cancer risk factors for sporadic and familial breast cancer may be elucidated through studies of women with BRCA1 mutations or in women in high risk breast/ovarian cancer families. There is variable penetrance for breast cancer among women in families with known BRCA1 mutations, and we hypothesize that this might be due to genetic variants in wild-type BRCA1 or other DNA repair genes and we hypothesized that these genetic variants might also affect sporadic breast cancer risk. The purpose of this study is to identify genotype-phenotype relationships in DNA repair-related genes using EBV-immortalized cell strains in women by assessing radiation-induced DNA repair capacity. Seventy-five affected and 57 unaffected BRCA1 mutation carriers with immortalized cell lines were analyzed in this study. Radiation-induced chromosomal breaks were measured as a marker of DNA repair capacity and the method was validated by assessing intra-individual variation in EBV-immortalized and peripheral blood lymphocytes. Concordant samples were analyzed and the Pearson correlation was found to be high ($r^2=0.865$, $p=0.01$). There was inter-individual variation in mean breaks per cell among the 132 women. Affected women were 3 times more likely to have higher mean breaks per cell than unaffected women. When unconditional logistic regression was done, no association between BRCA1 mutations, polymorphisms, haplotypes, or BRCA2 polymorphisms and DNA repair efficiency was found. Subjects with high mean breaks per cell were 2-fold more likely to be diagnosed with infiltrating ductal carcinoma and 4 times more likely to be diagnosed with metastatic breast cancer compared to subjects with low mean breaks per cell. The variable penetrance for breast cancer risk among BRCA1 carriers has not been explained by common polymorphisms in BRCA1 or BRCA2, or haplotypes of BRCA1 in this model.

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Introduction

Breast cancer is the second leading cause of cancer death and is the most common cancer in women living in the US (Greenlee et al.). Breast cancer risk for both sporadic and familial breast cancer can be better elucidated in some women through the study of cancer risk in women from high-risk families. In 1994, *BRCA1* was isolated (Miki et al.) and it is now recognized that women with *BRCA1* mutations have an increased risk of invasive breast and ovarian cancer. Specifically, inherited mutations in *BRCA1* are associated with a 56-87% risk of breast cancer before the age of 70 (Ford et al.;Struewing et al.), the lower estimate or risk representing an unselected population of individuals and the higher values are those with a strong family history of cancer. However, the determinants of why some women with mutations develop breast cancer (i.e. the penetrance) and others do not is mostly unknown. We hypothesize that genetic polymorphisms play an important role in both sporadic and hereditary breast cancer risk. The factors that regulate who actually gets breast cancer in women with highly penetrant mutations are only now being identified and it is possible that the same genetic traits and exposures trigger breast cancer in the general population; *BRCA1* mutation carriers are just substantially more susceptible to these gene-environment interactions. The study of penetrance in *BRCA1* mutation carriers, therefore, might identify potential sporadic breast cancer risk factors and provide for prioritization for study in the general population. Although, it is generally believed that this is due to dietary, lifestyle and environmental exposures, heritable traits and genetic susceptibilities may play a significant role. There are countless genetic polymorphisms that may modulate hereditary and sporadic breast cancer risk, but little guidance on which ones to study. Many polymorphisms have been identified, but the relationship between the allelic variant and its possible change in protein function is usually unknown. This study will utilize a unique resource and capitalize on a high risk group of women to provide important information about high-risk and sporadic breast cancer. Specifically, this study will be able to answer these questions:

1. Why do only some women that harbor the BRCA1 mutation get breast cancer and others don't?
2. Are there polymorphisms in (involved in DNA repair) genes that increase susceptibility or modify breast cancer risk in this group?
3. How would this apply to sporadic breast cancer patients? Will that polymorphism increase risk of breast cancer in the general population?

The significance of this study lies in ascertaining how polymorphisms in DNA repair genes affect *BRCA1* penetrance and how this information could be applied to sporadic breast cancer. This will allow for prioritizing which genes to assess in epidemiological studies, and also provide an indication about which ones might have the greatest effect and interaction with *BRCA1*. The identified polymorphisms will then be tested in an existing epidemiological breast cancer study, a funded NIH and DOD case-control study of breast cancer from the University of Buffalo that studies the relationship between alcohol, diet, oxidative damage and sporadic breast cancer. Ultimately, these studies will identify susceptible groups of women and aid in the development of more rationale prevention strategies. The aims and hypotheses of this study are listed below:

Aim 1: To identify DNA repair phenotypes in affected and healthy *BRCA1* mutation carriers that represent complex low penetrant genotypes by studying DNA repair proficiency in the terms of

chromosomal breaks in EBV-immortalized lymphocytes following *in vitro* treatment with gamma irradiation.
Hypothesis 1: There will be a range of responses, i.e. inter-individual variation, for chromosomal breaks using gamma irradiation among affected and unaffected women with *BRCA1* mutations.

Aim 2: To identify single nucleotide polymorphisms (SNPs) in *BRCA1*, *BRCA2*, and *Rad5*1 that are associated with defective DNA repair capacity in EBV-immortalized lymphocytes.
Hypothesis 2: There will be polymorphisms in DNA repair genes (*BRCA1*, *BRCA2*, *Rad51*) that will affect the number of chromosomal breaks in affected and healthy *BRCA1* mutation carriers

Aim 3: To identify single nucleotide polymorphisms (SNPs) in *BRCA1*, *BRCA2*, and *Rad5*1 associated with increased risk to sporadic breast cancer.
Hypothesis 3: Some of the single nucleotide polymorphisms shown in the previous aim to increase risk in hereditary breast cancer will be the same polymorphisms found to increase risk in cases versus controls.

Secondary Aim:
Aim 1: To compare the number of induced chromosomal breaks in EBV-immortalized and freshly cultured lymphocytes from affected and unaffected *BRCA1* carriers.
Hypothesis 1: Mutagen sensitivity in freshly cultured lymphocytes will be correlated with mutagen sensitivity in immortalized lymphocytes from the same women.

1. 0 DNA Repair Pathways

When DNA is altered or damaged, the cell can undergo apoptosis or cell arrest so the lesion can be repaired. DNA repair cannot only prevent mutations from being replicated, but it necessary to avoid interference with transcription. For these reasons, cells have developed clever ways to repair virtually every different kind of DNA lesion and at least four pathways of DNA repair can operate on specific types of damaged DNA. Each pathway is activated by different damaging agents and this is illustrated in **Figure 1**.

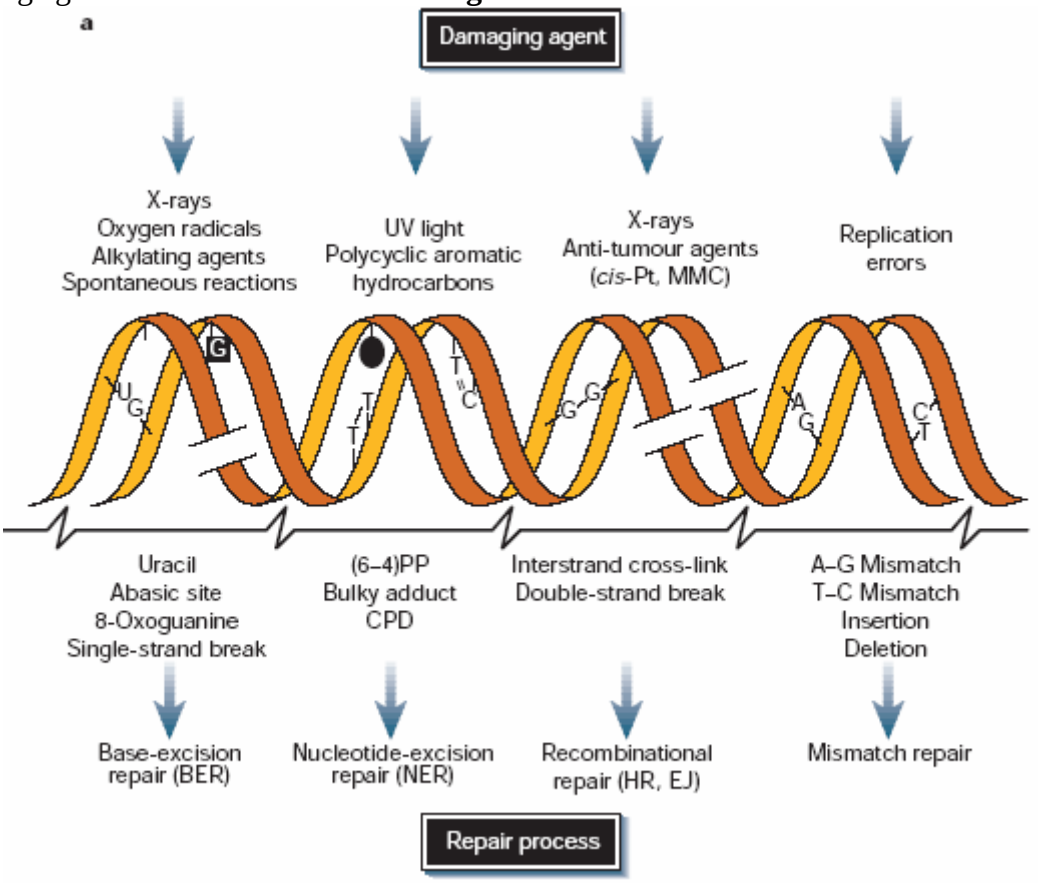


Figure 1. Common DNA damaging agents, examples of DNA lesions induced by agents, and most relevant DNA repair mechanism responsible for removal of lesion. Adapted from Hoeijmakers, *Nature*, 2001.

Base-excision repair (BER) operates on small lesions such as fragmented or non-bulky adducts, or those produced by methylating agents. It also defends against damage caused by oxygen radicals, spontaneous deamination, and hydrolysis. It is mostly responsible for recognizing minor damage to bases and sugars in DNA. The molecules involved in BER often recognize one substrate, remove the damage, and leaves a sugar phosphate backbone (abasic site). The APE1 endonuclease protein can cleave the abasic site, but single stranded breaks (SSBs) in the DNA strands are recognized and bound by PARP and it involves other proteins such as XRCC1. After XRCC1 binding or APE1 cleavage one of two pathways is activated: short patch BER or long patch BER. In short patch BER, the main pathway, damaged bases are replaced using XRCC1, DNA polymerase beta, and DNA ligase 3. Alternatively, in long patch BER, few bases are excised and removed by FEN-1, including bases adjacent to the damaged base, and incorporation of new nucleotides are mediated by PCNA, Polymerase delta or epsilon and DNA ligase I.

The nucleotide-excision repair (NER) pathway repairs bulky lesions such as pyrimidine dimers, other photo-products, larger chemical adducts, and DNA intra-strand cross-links. Briefly, there are also two different sub-pathways involved in NER and categorized by the way they recognize DNA damage. During global genome NER, DNA helix distortion is performed by XPC. In transcription coupled NER, the DNA lesion causes the DNA transcription to halt, and the RNA polymerase II complex allows for the recruitment of other NER proteins to remove the transcription blockade. It is after the recognition of the lesion, that the two pathways become one and now 25 or more NER proteins are recruited to the site of the lesion. The TFIIH transcription factor, which includes XPB and XPD, is required to open up the structure around the lesion and then RPA (replication protein A) coats the DNA of the undamaged strand, while NER endonucleases ERCC1/XPF and XPG cut the damaged strand 5' and 3' of the damaged base respectively. Ultimately, 20-30 oligonucleotides containing the damage are removed, then the NER protein complex in disassembled, and the regular DNA replication machinery is recruited to fill in the gap.

Another category of DNA repair is mismatch repair, which corrects replications errors caused by DNA polymerase (Aquilina and Bignami). In the mammalian system, the MSH2-MSH6 heterodimer recognizes base mismatches and loops containing 1-2 displaced bases, while the MSH2-MSH3 heterodimer has a preference for larger loops. After mismatch recognition, hMLH1-hPMS2 bind the site of repair where the complexes interconnect with other endo- and exonucleases needed for strand discrimination and for repair. Strand discrimination may be based on localizing the nearest replicating DNA polymerase. Excision of a stretch of DNA (~1kb) including the lesion follows and DNA resynthesis occurs to fill in the gaps.

1.1 Double Stranded Break Repair in Eukaryotes

Double-stranded breaks (DSB) can be produced by replication errors and by exogenous agents such as ionizing radiation or certain chemotherapeutic drugs, from endogenously generated reactive oxygen species; they can also be produced when DNA replication forks encounter DNA single-strand breaks (Khanna and Jackson). There are two different pathways involved in double-strand break repair: homology-directed repair (HR) and non-homologous end joining (NHEJ).

1.1.1 Non homologous end joining and Homologous Recombinational Repair

In non-homologous end joining repair, the two double-strand-breaks are directly ligated and in HR, the DNA ends are first resected in the 5' to 3' direction by nucleases; the resulting 3' single-stranded tails then invade the DNA double helix of a homologous, undamaged partner molecule, and are extended by the action of DNA polymerase, which copies information from the partner. In HR, the DNA ends are first resected in the 5' to 3' direction by nucleases; the resulting 3' single-stranded tails then invade the DNA double helix of a homologous, undamaged partner molecule, and are extended by the action of DNA polymerase, which copies information from the partner. *BRCA1* is involved in double-stranded break repair; there are two different pathways involved in double-strand break repair: homology-directed repair or homologous recombinational repair (HR) and non-homologous end joining (NHEJ). Both of these pathways and associated proteins are illustrated in Figure 2a and b.

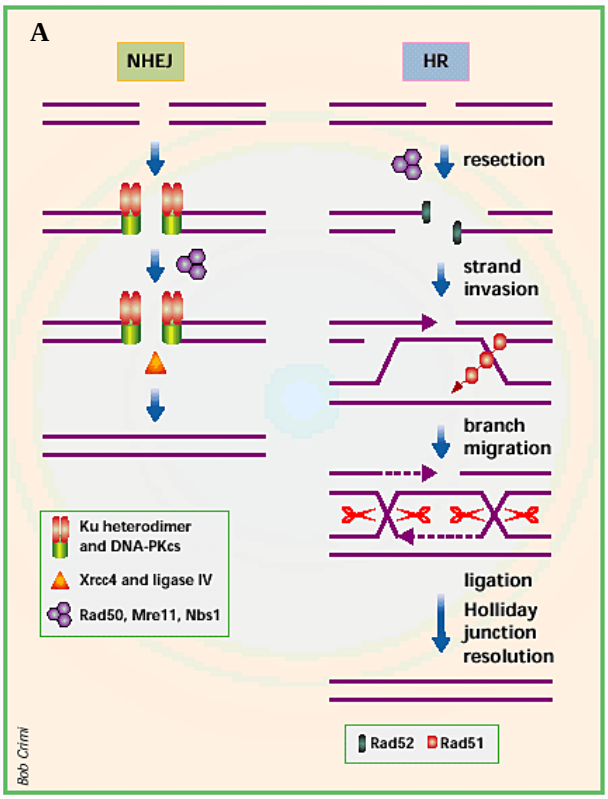


Figure 2a) (17): Pathways of DSB repair. NHEJ rejoins the two broken ends directly and generally leads to small DNA sequence deletions. It requires the DNA-end-binding protein Ku, which binds free DNA ends and recruits DNA-PKcs. Xrcc4 is then recruited along with DNA ligase IV. The Rad50-Mre11-Nbs1 complex, which contains helicase and exonuclease activities, may also function in NHEJ, particularly if the DNA ends require processing before ligation. HR requires Rad52, a DNA-end-binding protein, and Rad51, which forms filaments along the unwound DNA strand to facilitate strand invasion. The resected 3' end invades a homologous DNA duplex and is extended by DNA polymerase. In meiotic cells, the ends are ligated by DNA ligase I and the interwound DNA strands (Holliday junctions) are resolved resulting in either crossover or non-crossover gene conversion products. Only one of the many recombination products is shown here.

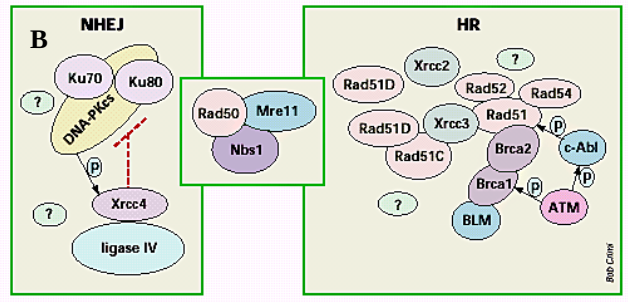


Figure 2b) (17): Components of DNA DSB repair pathways. NHEJ: Ku binds a DBS, followed by recruitment and activation of DNA-PKcs. XRCC4 and ligase IV are recruited directly or indirectly by the DNA-PK holoenzyme and/or are activated by DNA-PK–mediated phosphorylation. HR: proteins involved in mammals are indicated. The strand-exchange reaction catalyzed by Rad51 is facilitated by Rad52 through direct interaction. Rad54, a DNA-dependent ATPase, also interacts directly with Rad51 and stimulates its activity. Rad51-related proteins (Rad51B-D, Xrcc2 and Xrcc3) are also involved in HR. There is a direct interaction between Xrcc3 and Rad51, and Rad51B and Xrcc3 interact with Rad51C. Rad51 also interacts with Brca2 and indirectly with Brca1 through Brca2. The c-Abl tyrosine kinase modulates Rad51 strand exchange activity through phosphorylation. Brca1 and c-Abl are phosphorylated by ATM. The Mre11/Rad50/Nbs1 complex, which participates in both NHEJ and HR, is also indicated.

1.2 Proteins involved in Homologous Recombinational Repair

1.2.1 BRCA1

The function of *BRCA1* remains under active study. Many studies have proven that loss of BRCA1 protein results in defective DNA damage repair, abnormal centrosome duplication, cell-cycle arrest, growth retardation, increased apoptosis, genetic instability, and tumorigenesis, suggesting a role in DNA repair. Evidence is mounting that BRCA1 has local activities at double-stranded break (DSB) sites. Specifically, BRCA1 has a role in sensing DNA damage and checkpoint control of the cell cycle. It has been suggested that BRCA1's role in sensing breaks actually controls homology-directed DNA repair (Moynahan 1999). In particular, BRCA1 is rapidly phosphorylated, by the kinases ATM and CHK2 after DNA damage in dividing cells, suggesting that it may work downstream of checkpoints that sense and signal DNA damage or problems with DNA replication during S phase (Cortez et al.;Lee et al.;Tibbetts et al.). After phosphorylation, BRCA1 migrates to the site of the DSB, and the MRE11/RAD50/NBS1 complex is recruited (Wang et al.). The exonucleolytic activity of the complex is mediated by MRE11 and is responsible for resecting DSB ends to generate ssDNA tracts (Davies et al.).

Recent work suggests that BRCA1 regulates the activity of this complex and under certain *in vitro* conditions, BRCA1 can inhibit the activity of MRE11 (Paull et al.), regulating the length and the persistence of ssDNA generation at sites of DNA breakage.

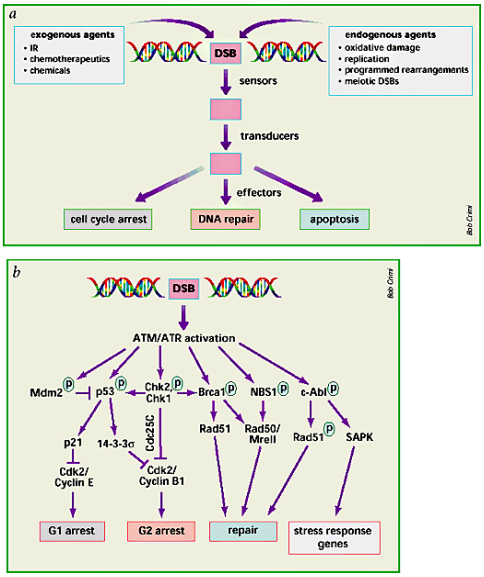


Figure 3 a) The general organization of the DNA-damage response pathway. The presence of DSBs is recognized by a sensor, which transmits the signal to a series of downstream effector molecules through a transduction cascade, to activate signaling mechanisms for cell-cycle arrest and induction of repair, or cell death if the damage is irreparable. **b)** ATM is activated in response to DSBs by an unknown mechanism. Activated ATM signals the presence of DNA damage by phosphorylating targets involved in cell-cycle arrest, DNA repair and stress response. In the DNA repair pathway, BRCA1 is phosphorylated and then signals BRCA2 (not shown), Rad51, Rad51/MRE11 to begin repair.

Additionally, interaction of BRCA1 with BRCA2 was suggested by the discovery that both proteins interact with RAD51 (Scully and Livingston;Welcsh, Owens, and King). Whereas it seems that RAD51 interacts with BRCA2 directly, the association of BRCA1 with RAD51 may be indirect, perhaps mediated by BRCA2 (Welcsh, Owens, and King).

1.2.2 BRCA2

The gene product of BRCA2 promotes and regulates the homologous recombination pathway of DNA double-strand-break repair (Davies et al.;Moynahan, Cui, and Jasin;Xia et al.). BRCA2 has been linked to double-stranded DNA repair because of its interaction with Rad51, a RecA homologue in yeast whose function has been determined to be in homology-directed DNA repair. The regions that of BRCA2 that interact with Rad51 have been mapped to 8 BRC repeats (Chen 1998, Wong1997). The 8 repeats in BRCA2 appear to be redundant for Rad51 binding, because any of the repeats bind Rad51 efficiently.

1.2.3 Rad51

RAD51 forms foci in the nucleus after irradiation and catalyzes strand exchange, an early step in homologous recombination that results in the formation of heteroduplex DNA molecules (Tashiro et al.). The three proteins (RAD51, BRCA1 and BRCA2) colocalize in irradiation induced foci (IRIF) and, notably, cells deficient in BRCA1 and BRCA2 are defective in RAD51 IRIF formation (Yu et al.;Yuan et al.;Bhattacharyya et al.;Huber et al.). Furthermore, it has also been shown that *BRCA1* deficient embryonic stem cells have decreased homologous recombinational repair (Koller 1999).

The relationship between BRCA1, BRCA2, and Rad51 is still actively being investigated, and in the last year, many studies have elucidated this complex's role in homologous recombinational repair. Because of the possible consequences of genetic polymorphisms in *BRCA1*, *BRCA2*, and *Rad51*, such as protein functional changes, this study will only focus on genetic polymorphisms of those genes.

2.1 Mutagen Sensitivity Assay and DNA repair

Suboptimal or deficient repair of DNA damage may be a susceptibility factor predisposing women to breast cancer through increased sensitivity to carcinogenic damage from environmental exposures. The mutagen sensitivity assay, also known as the radiation-induced G2 chromatid assay, has been proposed as a phenotypic assay for cancer risk, including breast cancer (Bell et al.). It is presumed that this assay is effective in measuring DNA repair capacity, at least in part. The assay measures defective DNA repair capacity by quantitating chromosomal breaks induced by gamma irradiation in cultured peripheral blood lymphocytes (PBL)(Sanford et al.;Parshad, Sanford, and Jones). Although many mutagens such as bleomycin, x-rays, and UV light have been used in its place, gamma radiation has been used for breast cancer studies and triggers a variety of DNA damage relevant to this proposal.

This assay, in small studies has been applied to high-risk families. For example, in cancer-prone individuals, cells display an abnormally high, at least two-fold higher number of breaks (Parshad and Sanford). Also, Sanford et al have shown in several studies that more than 60 breaks and gaps per 100 metaphase cells, or 0.6 mean breaks and gaps per cell, is considered to be suboptimal repair (Sanford et al.;Sanford et al.). In another study, it was found that women at high risk (having 1 or more first-degree relative with breast cancer) were 5 times more likely than controls to have suboptimal DNA repair (OR=5.2, 1.04-28.57). Deficient DNA repair was found in all women with breast cancer, but in only 32% of control women (p=0.02)(Helzlsouer et al.).

Several groups doing the mutagen sensitivity assay have found a significantly increased incidence of sensitivity compared with controls. In one of the largest epidemiological studies, it was found that when bleomycin (BLM) was used, 97% of hereditary breast cancer cases, 30% of their family members, 44% of sporadic cases, and 21% of controls were either sensitive or hypersensitive (>0.8 mean breaks per cell) (Jyothish et al.). In other studies gamma radiation induced a higher degree of chromosomal damage in lymphocytes of family members of breast cancer patients compared with controls. In general, many other studies that have concluded ineffective DNA repair may be associated with cancer susceptibility in breast cancer patients and their first-degree relatives (Roy et al.;Patel et al.;Parshad et al.;Knight et al.;Pathak et al.;Pero et al.). Table 1 lists all of the mutagen sensitivity studies relevant to this study.

Table 1. Mutagen Sensitivity Studies

Author	Year	Mutagen	Normal (mbc)	Tumor (mbc)	Odds Ratios
Knight	93	X-Ray	0.38-0.48	1.1-1.9	
Helzsouer	95	X-Ray	0.2-0.28	0.22-1.28	
Helzsouer	96	X-Ray	0.16-0.5	1.1-1.6	
Parshad	96	X-Ray	0.4	0.9-0.70	2.1-622.1
Patel	97	X-Ray	0.6-0.92	1.05-1.59	6.9-23.8
Jyothish	98	Bleomycin	0.58	0.8-1.28	1.3- 41.3
Roy	2000	Bleomycin			4.1
Hsu	85	Bleomycin	12%>1 MBC	60% >1 MBC	11.6
Scott	94	X-Ray	0.94	1.09	6.9

Key Research Accomplishments, Reportable Outcomes, and Conclusions

Aim 1: To identify DNA repair phenotypes in affected and healthy *BRCA1* mutation carriers that represent complex low penetrant genotypes by studying DNA repair proficiency in the terms of chromosomal breaks in EBV-immortalized lymphocytes following *in vitro* treatment with gamma irradiation.

Hypothesis 1: There will be a range of responses, i.e. inter-individual variation, for chromosomal breaks using gamma irradiation among affected and unaffected women with *BRCA1* mutations.

Subjects: The subjects in this study are both confirmed BRCA1 mutation carriers and non-carriers recruited through the Lombardi Comprehensive Cancer Center –Familial Cancer Registry (LCCC-FCR). Of the subjects analyzed (n=135), 75 are affected and 60 are unaffected (healthy) *BRCA1* mutation carriers. Peripheral blood lymphocytes were immortalized by EBV and stored in liquid nitrogen by our tissue culture core. Thirty-four patients without founder mutations (187delAG and 5382insC) had complete *BRCA1* and *BRCA2* gene sequencing. If the patient was Jewish, then they were only tested for the 3 founder mutations. If the patient was not Jewish, then Myriad did full sequencing. If a non-Jewish patient has a relative that has already tested positive for a BRCA1 mutation, then the patient is only tested for the mutation that has already been identified in her relative. Some patients are related (n=56). Patient demographics are listed on **table 2**.

Table 2. Patient Demographics (only available information analyzed).

Characteristic		With Cancer	Without Cancer	
		n=86	***n=55***	
Age Range (yr)		27-71	22-78	
Median Age (yr)		47	45	
	SD	10.95	13.55	
Mean Age (yr)		45.96	44.97	p=0.43
Race				
	White	91 (92%)	53 (98%)	
	Black	5 (5%)	0	
	Hispanic/Latino	2 (2%)	1 (2%)	
	Unknown	1 (1%)	0	
Mutation				
	185delAG (BRCA1)	32 (32%)	22 (41%)	
	5382insC (BRCA1)	14 (14%)	7 (13%)	
	Other	53 (54%)	25 (46%)	

Cell Lines: To date, 168 EBV-immortalized cell lines have been obtained from Georgetown University's Tissue Culture Core. The Tissue Culture Shared Resource (TCSR) exists to assist the Cancer Center investigators and other members of the Georgetown University research community with the tissue culture related aspects of their research. The TCSR manages

Lombardi's cell line repository, which includes an extensive collection of normal and tumor cell lines (animal and human) stored in a series of liquid nitrogen freezers. These cell lines are made available to investigators either as frozen vials of cells or as growing cultures so our cell samples were either delivered in a flask containing live cells in 10 mL of media or as a frozen cell pellet containing around 1 x 10^6 cells. The TCSR, also, obtains fresh blood from BRCA1 mutation carriers and provides EBV immortalization of B cells. All samples were labeled and logged in a secure database.

Of those 168 cell lines obtained, 20 were BRCA1/2 negative, 3 were males, and 8 were not Caucasian. The males and subjects that were not Caucasian were not included in our study. Also, of those cell lines obtained, 16 died, and 2 did not produce enough metaphases, and also were not analyzed in our study. Of the 135 total that were be analyzed, 56 were related.

Along with the 168 cell lines, 20 fresh blood samples have also been received, labeled, and logged into a secure database. Of the 20 fresh blood samples, 19 corresponding cell lines have been collected for the comparison between fresh blood and EBV-immortalized cell line mutagen sensitivity. One other corresponding EBV-immortalized cell line is still undergoing transformation. See appendix A for list of cell lines received.

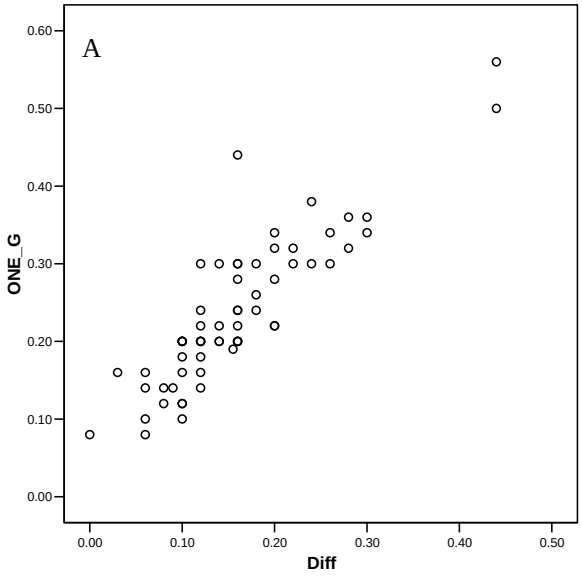
Mutagen Sensitivity: This project began by characterizing DNA repair capacity in healthy and affected subjects using immortalized lymphocytes and determining their sensitivity to mutagen exposure. The assay is called "mutagen sensitivity assay" and it is a marker of DNA repair. The mutagen sensitivity assay has been performed on 152 EBV-immortalized cell lines and all 20 fresh blood samples. In short, 1Gy radiation from a ^{137}Cs source gamma irradiator was used to induce chromosomal breakage according to the method developed by Sanford and Parshad (Parshad, Sanford, and Jones;Sanford et al.). The culture medium used was RPMI1640 with L-glutamine. Fresh media was made once a week. Cultures were incubated for 4 hours to allow time for repair of breaks induced in the chromosomes. The cultures were then treated with colcemid to accumulate mitosis before harvesting. The cultures were then fixed, 4 slides per sample prepared (2-OGy and 2-1Gy), and stained with Giemsa. Chromosomal breaks were scored by microscopy. The frequency of chromatid breaks per cell (b/c) was calculated as a measure of an individual's DNA repair efficiency. Only chromatid breaks (discontinuity longer that the chromatid width) was scored. A minimum of 25 and a maximum of 50 metaphases per culture were scored. The polymorphisms that are found to affect mutagen sensitivity have been used to determine if there is indeed a genotype that is associated with breast cancer risk.

Reportable Outcomes: Aim 1

Preliminary Results:

Initially, when performing the mutagen sensitivity assay on cell lines, mutagen sensitivity (or DNA repair capacity) was determined by subtracting baseline mean breaks per cell (0G- non-irradiated control slides) from radiation treated slides (1G). It was observed that most non-irradiated slide values were close to zero and thus a correlation analysis between irradiated (1G) and irradiated minus non-irradiated (1G-0G) values was determined. The values were highly correlated; Pearson correlation: r^2=0.87, $p<0.01$ (**figure 4**). With this new information, we revised our plan of subtracting baseline values from irradiated values to determine mutagen

sensitivity and determined that mutagen sensitivity could be determined by only considering the irradiated values.

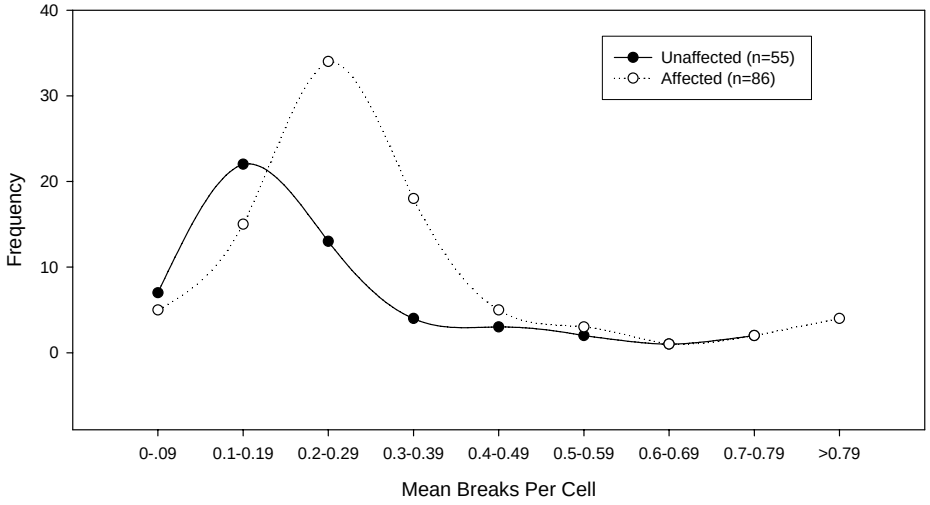


B	Mean	Range
0 Gy	0.08	0-0.18
1 Gy	0.26	0.08-1.23
1G-0G	0.20	0.06-1.13

Figure 4. a) Comparison of radiation-treated slide vs. Difference between radiation-treated minus non-irradiated slides. b) Mean and ranges of treated and non-treated slides.

Results and Statistical Analysis: The distribution of chromatid breaks are described in **figure 5**. A frequency graph of 141 samples was plotted against the number of breaks. Only data analyzed is included in the figure. The independent variable is the frequency of mean breaks per cell and the dependent variable is breast cancer being affected or healthy and frequency.

Figure 5. a) Frequency of mean breaks per cell in 45 cell lines in affected BRCA1 mutation carriers compared to healthy BRCA1 mutation carriers.



When unconditional logistic regression was done to determine if there was an association between mean breaks per cell and being affected or unaffected with cancer, it was found that subjects with higher mean breaks per cell (determined by the median value of the unaffected)

were 3 times form likely to be affected with breast cancer. When the median mean breaks per cell of the whole sample was considered, subjects with higher mean breaks per cell were still more likely to be affected with breast cancer (**figure 6**). These results are consistent with the studies listed in **table 1.**

Figure 6. Mutagen sensitivity in unaffected subjects compared to affected subjects. a) High was determined by unaffected median. $\chi 2$= 7.1, p=0.0077. b) High was determined by sample median; p<0.01

A	Mutagen Sensitivity (MBC)			
Mean Breaks per Cell	Unaffected (N=55)	Affected (N=87)	OR	95% CI
Low *	25	18	1.0	
High	30	69	3.2*	1.5 - 6.7

B	Mutagen Sensitivity (MBC)			
Mean Breaks per Cell	Unaffected (N=55)	Affected (N=87)	OR	95% CI
Low *	37	40	1.0	
High	18	47	2.4	1.2 - 4.9

Epidemiological Statistical Analysis

Since the majority of the subjects had taken an epidemiological questionnaire prior to blood draw, unconditional logistic analysis was done for certain risk factors to see if there was any association between mean breaks per cell (phenotype) and risk factors. Of all the risk factors assessed (such as radiation therapy, chemotherapy, hormonal factors such as menses, child bearing, etc) only 2 interactions emerged. Breast cancer pathology seemed to be well correlated with high mean breaks per cell. Although marginal, subjects with high mean breaks per cell were almost 2 times more likely to have infiltrating ductal carcinoman ($\chi 2$= 2.34 p=0.12; OR: 1.8 CI:0.9-3.6); subjects with metastatic breast cancer were almost 4 times more likely to have higher mean breaks per cell ($\chi 2$=6.69, p=0.009; OR: 3.9 CI: 1.3-11.6).

Reportable Outcomes and Conclusions for Aim 1

- There are a range of responses in patient population, consistent with hypothesis 1
- BRCA1 affected cases have a 3.2-fold chance of having higher mean breaks per cell compared to BRCA1 unaffected carriers, consistent with literature
- Breast Cancer pathology (infiltrating ductal and metastatic disease) may be correlated with mutagen sensitivity phenotype

Secondary Aim 1: To compare the number of induced chromosomal breaks in EBV-immortalized and freshly cultured lymphocytes from affected and unaffected *BRCA1* carriers.

Secondary Hypothesis 1: Mutagen sensitivity in freshly cultured lymphocytes will be correlated with mutagen sensitivity in immortalized lymphocytes from the same women.

Reportable Outcomes and Conclusions for Secondary Aim 1

I am currently working on journal article manuscript and will be submitted in the near future. Un-submitted manuscript with results and conclusions can be found in Appendix A.

Aim 2: To identify single nucleotide polymorphisms (SNPs) in *BRCA1*, *BRCA2*, and *Rad5*1 that are associated with defective DNA repair capacity in EBV-immortalized lymphocytes.

Hypothesis 2: There will be polymorphisms in DNA repair genes (*BRCA1*, *BRCA2*, *Rad51*) that will affect the number of chromosomal breaks in affected and healthy *BRCA1* mutation carriers.

DNA Extractions: DNA from cell culture pellets has been extracted by the Qiagen M48 Biorobot. In short, cell culture pellets were collected, washed in PBS, and stored in -20°C until time for extraction. All of the samples were carefully labeled and logged into a secure database. One day before extraction, approximately $2.0x10^6$ cells were digested with 10uL of *proteinase K*, to degrade unwanted proteins and incubated in a 55°C water bath. After digestion, 150uL of PBS was added and samples were loaded in the M48 Biorobot (48 samples at a time). After extraction, DNA samples were quantitated by Genequant or Spectramax. Acceptable DNA:Protein (ratios) are 1.7-1.9, and if the quality was found to be outside these parameters, DNA was extracted again. After DNA was quantitated, aliquots of 50ug/ul, 10ug/ul, and 2ug/ul were made, labeled and logged into a secure database. DNA has been extracted and purified from: 16 Lows (≤0.1 mean breaks per cell); 22 Highs (≥0.4 mean breaks per cell); 18 from in between Low and High group; 56 DNA samples total (80 total with repeats).

**On March 1, decided to extract all DNA- in process

Genotype/Haplotype analysis: Polymorphisms worth characterizing and analyzing were identified in a preliminary study of sequencing data by Myriad Genetic Laboratories, Inc. in 36 patients without founder mutations (187delAG and 5382insC) and from the scientific literature and various web-based resources such as NIH's dbSNP and SNP500. These SNPs (P871L, E1038G, S1613G, K1183R) were chosen to be analyzed because they were the most frequently found SNPs in 36 *BRCA1* mutation carriers and because they were found in an important region were an amino acid change could lead to change in the structure and function of the protein. PCR-based genotyping assays were going to be developed for *BRCA1*'s P871L, E1038G, S1613G, K1183R, but it was determined that they were all found in 1 haplotype block and by testing only 1 SNP, we would be informed about the other SNPs in the same block (**figure 7**). It was also determined that it would be important to do haplotypes analysis for the whole *BRCA1* gene, given that 7 genotypes/haplotypes would inform us about 26 other SNPs. Real time PCR assays are currently being developed for this aim.

Statistical Analysis of Genotyping Data: With the sequencing data obtained from Myriad, preliminary analyses of genotype-phenotype correlations were done. The association between genotype and the mean number of breaks per cell has been calculated by unconditional logistic regression and odds ratio of relative risk with 95% confidence intervals was calculated. Results of logistic regression analyses have shown that **there is no association between any BRCA1 SNP and phenotype**. Even after adjusting for confounders such as age and mutation, there was no association. SNPs to further assess in a case-control study will be determined by the results of statistical analysis.

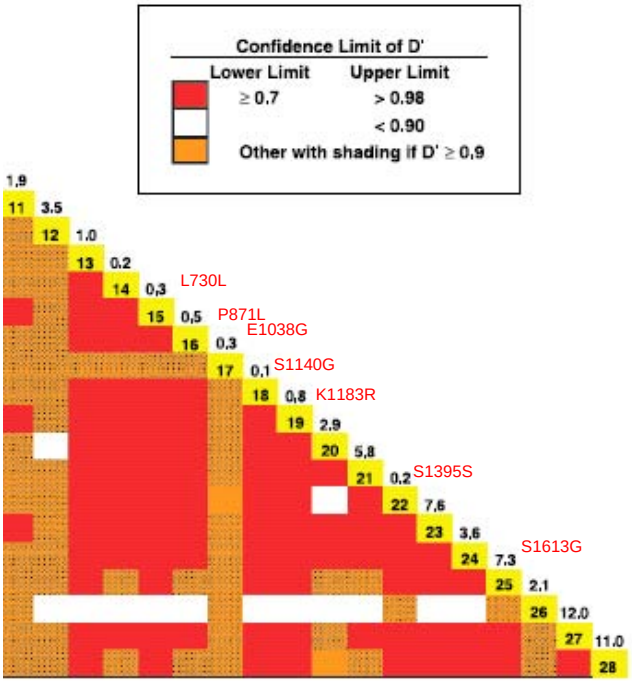


Figure 7. BRCA1 haplotype Block 2 includes SNPs L730L, P871L, E1038G, S1140G, K1183R, S1395S, and S1613G. All of these SNPs are in high linkage disequilibrium. Adapted from Freedman et al, 2005.

Reportable Outcomes and Conclusions for Aim 2

- No association between Mean Breaks per Cell and Haplotype has been found (n=32)
- DNA extractions are being performed on all subjects to do haplotypes analysis in this population
- 7 real time PCR assays will be developed for haplotypes analysis

Rad51 SNP discovery and analysis:

SNP discovery in Rad51 has been done by direct sequencing of four functionally relevant exons. Exon 4 is being sequenced because it contains a DNA binding domain and binds other Rads at this location. Exon 7, also binds other Rads at this location, binds DNA, and binds the BRC repeats of BRCA2. Exons 8 and 9, also bind the BRC repeats of BRCA2. Exon functional information and "established" variants that have been identified are listed in **Table 3**.

VariantSEQr Resequencing System primers were purchased from Applied Biosystems. The system is designed for resequencing the exons of genes implicated in cancer and various other complex diseases. VariantSEQr Resequencing System provides ready to use primer sets and a universal protocol for PCR, sequencing, and data analysis. In short, genomic DNA was amplified with a primer set, Amplitaq Gold PCR Master mix, ddH20, and 50% glycerol. The thermal cycler was programmed for activation at 96°C for 5 minutes, 40 cycles of amplification at 94°C for 30 seconds, 60°C for 45 seconds, and 72°C for 45 seconds. The final extension was done at 72°C for 10 minutes. After amplification, the sample is held at 4°C indefinitely.

After the PCR, the reaction was cleaned up using ExoSAP-it. In short, 4ul of ExoSAP-it was added to 10uL of PCR reaction. Then the reaction was centrifuged and placed in the thermal cycler for a program of 37°C for 30 minutes, 80°C for 15 minutes, and sample is held at 4°C

indefinitely. After clean-up, the DNA is quantitated by running 3ul of sample against a DNA mass ladder on 2% agarose.

For sequencing, BigDye Terminator Reaction mix is added to 100ng/ul of clean DNA, *M13* universal forward primer, and dH20. For this PCR reaction, the thermal cycler conditions are as follow: Denaturation at 96°C for 1 minute, 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minutes. After amplification, the sample was held at 4°C indefinitely.

Reactions were then purified by spinning in a 96-well sephadex spin column. After purification, samples were then loaded into the MagaBace capillary sequencer, where specific parameters are loaded for each reaction plate. Results were using the Sequencher software.

Results of Sequencing

Initially, we had chosen to do direct sequencing of 4 Rad51 exons (exons 4, 7, 8, and 9) in 40 subjects with the highest and lowest mean breaks per cell. No mutations of polymorphisms have been found or confirmed in these subjects. So, it was decided that all Rad51 coding exons should be sequenced (exons 2, 3, 4, 6, 7, 8, 9, 10) in subjects with highest and lowest mean breaks per cell.

Currently, I have successfully amplified 8 exons and all PCR products for 56 samples are ready for sequencing. Sequences will be analyzed using Sequencher software. Found SNPs and mutations will be confirmed by sequencing in the reverse direction.

Table 3. Rad51 Exon information and variants found.

Exon	Functions	Variants (rs#)	change?	base Δ	AA Δ	Seq.?
Promoter	has Sp1 sites	2619679				NO
Promoter	Has large CpG island	7171780				
Promoter		7171963				
Promoter		5030790				
1		1801320	untranslated	C>G		NO
1		1801321	untranslated	G>T		
1		2619681	untranslated	C>T		
1		2619680	untranslated	C>A		
1		3092981	untranslated	C>T		
2		NONE				NO
3	Phosphorylated by ABL	NONE				?
3	contains HhH DNA binding domain	NONE				
4	contains HhH DNA binding domain	7174493	non-synonimous	G>C	Val>Leu	YES
4	Binds other Rads					
5	modulates ATPase activity	NONE				NO
6	Binds other RAds	NONE				?
7	Binds other RAds	11544204	synonimous	G>A	Arg>Arg	YES
7	Bind BRC repeats of BRCA2	7161941	intronic	T>A		
7	Binds DNA	7183120	intronic			
8 and 9	Bind BRC repeats of BRCA2	2229876	intronic	T>A		
8 and 9	Phosphorylated by ABL	4423392	intronic			YES
8 and 9		1804269		C>T		
8 and 9		11544205	non-synonimous	T>G	Ser>Ala	
10		1056742	non-synonimous	C>A	Q>K	?
10		12593359	untranslated			
10		7180135	untranslated	G>A		
10		11855560	untranslated			
10		11852786	intronic			

Reportable Outcomes and Conclusions for Aim 2

- No Rad51 mutations or polymorphisms have been found or confirmed

Challenges and Obstacles:

The purpose of this project is to identify DNA repair gene polymorphisms in a "uniform" population of subjects, women with BRCA1 mutations, that would potentially result in risk modification in this population and could result in increasing or decreasing breast cancer risk in the general population. To date, no polymorphisms or haplotypes in *BRCA1* have been found to be correlated with the mean breaks per cell phenotype in this population. This could be attributed to a small sample size and we are now interested in haplotyping the whole hereditary population (n=132) to see any association before moving to aim 3. We, also, have not been able to identify any mutations or polymorphisms in Rad51 associated with mean breaks per cell. This could also be attributed, again, to a small sample size and as you can see in table 3, to date, very few polymorphisms have been found and confirmed in Rad51. My thesis committee and I have decided that if no associations between *BRCA1* and *Rad51* genotype and mean breaks per cell (phenotype) are found, it will not be worthy to move on to Aim 3, which was dependent upon Aim 2. In Aim 3, I had hypothesized that found SNPs in the hereditary population would also modify risk in the general population.

Aim 3**:** To identify single nucleotide polymorphisms (SNPs) in *BRCA1, BRCA2,* and *Rad*51 associated with increased risk to sporadic breast cancer. (This aim cannot be worked until aim 2 is complete).

Hypothesis 3: Some of the single nucleotide polymorphisms shown in the previous aim to increase risk in hereditary breast cancer will be the same polymorphisms found to increase risk in cases versus controls.

Subjects: Dr. Jo Freudenheim at the State University of New York at Buffalo, our collaborator, has enrolled 1165 breast cancer cases and 2170 controls in a breast cancer and alcohol case-control study. The women are all residents of Erie and Niagara counties, New York. Cases are women with incident breast cancer between the ages of 35-79 years. Controls were randomly selected from residents of Erie and Niagara Counties using lists provided by the New York State Department of Motor Vehicles driver's License enrollees for those less than 65 years of age, and the Health care Finance Administration for those 65 years of age and older. Controls were frequency matched by age, sex, race, and county of residences to cases. Blood and oral rinses from cases and controls have been obtained and DNA has been extracted by phenol-chloroform methods. DNA aliquots were made labeled, logged in a secure database, and store in -20°C.

Genotyping: Polymorphisms identified in hereditary cases (aim 2) will be used to genotype in this epidemiological case-control study. Assays will be developed and genotyping will be done by RFLP or direct sequencing. Twenty percent of the samples will be repeated for quality control.

Statistical Analysis: Allele frequencies in cases vs. control subjects will be compared using the chi squared test and a test of Hardy Weinberg equilibrium. The association of disease status and

polymorphisms will be analyzed using unconditional logistic regression. Odds ratio estimates of relative risk with 95% confidence intervals will be used in the statistical analysis of the data and will be adjusted for confounders.

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Appendix A: Unsubmitted manuscript

Mutagen Sensitivity in EBV-immortalized cell lines correlated to fresh peripheral blood lymphocytes using the G2 chromatid break assay

ABSTRACT

Deficient DNA repair may be a susceptibility factor predisposing women to breast cancer through increased sensitivity to carcinogenic damage from environmental exposures. The mutagen sensitivity assay, also known as the radiation-induced G2 chromatid break assay, has been proposed as a phenotypic assay for cancer risk, including breast cancer. Usually for this assay, fresh blood is used and often there is not enough subject sample left to reproduce and validate results and to allow additional molecular biological techniques, such as PCR. These shortcomings in our investigation can be circumvented by immortalizing or transforming and continuously growing human B-lymphocytes from blood, and infecting with EBV (Epstein-Barr Virus). Immortalizing the cells with EBV provides a valuable permanent source for almost unlimited amounts of DNA from almost any individual, required for epidemiological studies. To determine if EBV-immortalized cell lines could be used in place of fresh blood in the G2 chromatid break assay, we investigated whether radiation response or DNA repair capacity of EBV-immortalized lymphoblastoid cell lines is the same as in the concordant sample of resting fresh PBL. We found a strong correlation between mean breaks per cell in fresh peripheral blood lymphocytes and their matching EBV-immortalized cell line. Results were reproducible and variability was only found in subjects with highest mean breaks per cell.

Introduction

Suboptimal or deficient repair of DNA damage may be a susceptibility factor predisposing women to breast cancer through increased sensitivity to carcinogenic damage from environmental exposures. The mutagen sensitivity assay, also known as the radiation-induced G2 chromatic assay, has been proposed as a phenotypic assay for cancer risk, including breast cancer (Parshad, Sanford, and Jones;Sanford et al.). It is presumed that this assay is effective in measuring DNA repair capacity, at least in part. The assay measures defective DNA repair capacity by quantitating chromosomal breaks induced by gamma irradiation in cultured peripheral blood lymphocytes (PBL)(Sanford et al.;Parshad, Sanford, and Jones). In short, frank breaks are counted in metaphase cells where DNA damage was induced by a mutagen. Although mutagens such as bleomycin, x-rays, and UV light have been used in this assay, gamma radiation has been used for breast cancer studies and it is known to trigger a variety of DNA damage. Other assays such as the micronucleus and comet can also measure DNA repair, but this paper will only focus on the G2 chromatic break assay or the mutagen sensitivity assay.

In small studies, the G2 chromatic break assay has been applied to high-risk families. For example, in cancer-prone individuals, cells display an abnormally high, at least two-fold higher, number of breaks (Parshad and Sanford). Also, Sanford et al have shown in several studies that more than 60 breaks and gaps per 100 metaphase cells, or 0.6 mean breaks and gaps per cell, is considered to be suboptimal repair (Sanford et al.;Sanford et al.). In another study, it was found that women at high risk (having 1 or more first-degree relative with breast cancer) were 5 times more likely than controls to have suboptimal DNA repair (OR=5.2, 1.04-28.57). Deficient DNA repair was found in all women with breast cancer, but in only 32% of control women (p=0.02) (Helzlsouer et al.).

Several groups doing the mutagen sensitivity assay have found a significantly increased incidence of sensitivity compared with controls. In one of the largest epidemiological studies, it was found that when bleomycin (BLM) was used, 97% of hereditary breast cancer cases, 30% of their family members, 44% of sporadic cases, and 21% of controls were either sensitive or hypersensitive (>0.8 mean breaks per cell) (Jyothish et al.). In other studies gamma radiation induced a higher degree of chromosomal damage in lymphocytes of family members of breast cancer patients compared with controls. In general, many other studies that have concluded ineffective DNA repair may be associated with cancer susceptibility in breast cancer patients and their first-degree relatives (Roy et al.;Patel et al.;Parshad et al.;Knight et al.;Pathak et al.;Pero et al.).

Previously, studies using the mutagen sensitivity assay to determine cancer risk used freshly drawn peripheral lymphocytes (PBL). When fresh peripheral blood lymphocytes (PBLs) are used, the volume obtained is usually enough for one experiment. Ultimately, the assay cannot be repeated unless the there is enough to set up a duplicate culture. Also, the short supply of PBL will not allow additional molecular techniques, such as PCR, to be applied. Cryopreservation of lymphocytes has also been used when the availability of subject samples are unpredictable and is useful for preserving valuable specimens. A comparison of fresh blood and cryopreserved lymphocytes showed that inter and intra-individual variability was high and experimental failures were high and that mutagen sensitivity in cyropreserved lymphocytes is higher than mutagen sensitivity in fresh blood. These shortcomings can be circumvented by immortalizing or transforming and continuously growing the cells under investigation. Human B lymphocytes can be immortalized with infection by EBV (Epstein-Barr Virus) in vitro as well as

in vivo. Immortalizing the cells with EBV provides a valuable permanent source for almost unlimited amounts of DNA from almost any individual, like it is required for epidemiological and hereditary studies. EBV is able to immortalize resting B cells and allow lymphoblastoid cell lines (LCLs) to proliferate continuously, ultimately leading to an endless supply of monoclonal B-cell lymphocytes. Usually, primary cell cultures (non-transformed) have a lifespan of only weeks or months and senesce at 50-60 doublings (Hayflick's number). With EBV immortalization, a culture is capable of greater than 150-200 doublings and is considered immortal. They can also grow continuously for more than a year and there is indefinite storability in liquid nitrogen and recoverability. In general and under optimal cell culture conditions, EBV-immortalized cell lines stably retain the major features of their original cell lines. Although genetic alterations occur, these genetic changes presumably provide the affected cell with either proliferative or survival advantages, and may play an important role in the *in-vitro* establishment of the cell line .

In this study, we wanted to investigate whether the mutagen response of an EBV-immortalized lymphoblastoid cell line is the same as in the concordant sample of resting fresh PBL. For this study, the mutagen sensitivity assay was performed on EBV-transformed lymphoblastoid cells of patients, some with *BRCA1* mutations, as well as their corresponding fresh PBLs.

Materials and Methods

Blood Samples and Cell lines

Blood samples were obtained from 17 subjects (Table 1). Of the 17 subjects, 7 were cancer negative (unaffected) and 10 were breast cancer positive (affected). Of the unaffected, 6 were true negatives, not having *BRCA1/2* mutations and 2 had the 5382insC BRCA1 mutation.

In short, a blood draw was performed by GUMC-GCRC (Georgetown University Medical Center General Clinical Research Center) phlebotomist. The single blood draw produced 2 green-top tubes with 2-10 ml of blood of which one was delivered to our lab and one was delivered to the GUMC Tissue Culture Core (TCC). All EBV-immortalization was done by the tissue culture core, and was delivered by to us them after immortalization. Remaining blood was separated into red blood cells and white blood cells by centrifugation at 2200G for a minimum of 25 minutes in a CPT Tube, and lymphocytes were then separated and stored in the liquid nitrogen with 90% fetal bovine serum and 10% DMSO.

The lymphoblastoid cells were cultured at 37°C in 5% CO_2 in RPMI1640 medium (GIBCO) supplemented with 10% fetal calf serum (Sigma), 2% L-glutamine (GIBCO), 1% Sodium Pyruvate (GIBCO), 1% NEAA (non-essential amino acids-GIBCO), 0.1% 2-mercaptoethanol (GIBCO), and 0.1% gentamycin (Invitrogen). At the time of the assay, cells had undergone less than 15 population doublings.

The G2 chromatid break assay

The mutagen sensitivity assay has been performed on 17 EBV-immortalized cell lines and all 20 fresh blood samples. In short, 1Gy radiation from a ^{137}Cs source gamma irradiator was used to induce chromosomal breakage according to the method developed by Sanford and Parshad (Parshad, Sanford, and Jones;Sanford et al.). The culture medium used was RPMI1640 with L-glutamine. Fresh media was made once a week. Cultures were incubated for 4 hours to allow time for repair of breaks induced in the chromosomes. The cultures were then treated with colcemid (GIBCO) to accumulate mitosis before harvesting. The cultures were then fixed (3:1 methanol:acetic acid), 4 slides per sample prepared (2-O Gray and 2-1 Gray), and stained with

Giemsa for 10 minutes. Chromosomal breaks were scored by light microscopy. The frequency of chromatid breaks per cell (b/c) was calculated as a measure of an individual's DNA repair efficiency. Only chromatid breaks (discontinuity longer that the chromatid width) was scored. A minimum of 25 and a maximum of 50 metaphases per culture were scored. Because the mean breaks per cell at 0Gy were close to 0 (data not shown), we did not continue to read them and only considered 1Gy readings for this study.

Statistical Analysis

For the comparison of mutagen sensitivity responses, the spearman correlation was calculated. The coefficient of variation was also calculated to analyze the variabilities obtained in PBL and EBV-transformed cell lines.

Results

Intra-individual Variation

To test intra-individual (inter-culture) variation, separate EBV-immortalized cultures were set up for subjects on the same day or on different days. The cytogeneticist was blinded and did not know which slides belonged to which subject. The mean breaks per cell and standard deviations are listed in *Table 1.* Initially, 2 separate cultures were set up for 4 subjects and the mutagen sensitivity assay was performed on the same day (ρ=0.932; $p<0.01$) (Fig. 1a). Additionally, 10 cultures were set up, on day 1 and then set up again on day 4, day 8 (n=3), and day 12 (n=2) and results were then compared using the spearman correlation (ρ=1.0-0.82; p<.01-0.33) (Table 2 and Fig.1b).

Inter-individual Variation

The results indicate the mean breaks per cell in EBV-immortalized cell lines *was not* significantly higher than in fresh peripheral blood lymphocytes of the corresponding subjects (ρ=0.907; $p< 0.01$) and are summarized in Table 3 and presented graphically in Figure 2.

Cancer status and variability

Because some of the samples where from subjects that had breast cancer, we wanted to see if cancer status affected variation in concordant subject samples. EBV-immortalized cell lines and peripheral blood lymphocytes mean breaks per cell were stratified cancer status (affected vs. unaffected) and compared (Table and Fig. 4). Although unaffecteds had a lower mean breaks per cell than affected, there was still little difference between concordant subject samples. These results reveals that EBV-immortalized lymphocytes *do not* display increased chromosomal instability and thus, EBV-immortalized cell lines could be used in the mutagen sensitivity assay, if the corresponding fresh sample is not readily available.

Discussion

In this study and paired examination of DNA repair capacity was done in fresh peripheral blood lymphocytes and concordant EBV-immortalized cell lines. These results indicated that the G2 chromatid break assay is a suitable DNA repair assay with little intra-individual variability compared to other DNA repair assays. Assay reproducibility was demonstrated between samples and thus confirmed what has previously been shown (Vral 2002; Scott 98/99).

Even without this data, some have already done a mutagen sensitivity assay on EBV immortalized cell lines to detect radiosensitivity in patients vs. control (Sturgis 1999;Baeyens 2004)

However, this study has limitations. Only a small number of subjects were examined and it has been suggested the EBV transformation might alter radio- and mutagen sensitivity (Trenz 2002). It was also shown that the higher the mean breaks per cell, the higher the variability and because of this we suggest that those subjects with high mean break per cell be re-evaluated accordingly.

In conclusion, the results obtained in this study show that EBV-immortalized cell lines can be used in place of fresh peripheral blood lymphocytes because similar results were noted between concordant samples.

Acknowledgements

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Tables

Table 1. Mean Breaks per cell and standard deviation within each subject.

Subject ID	Mean Breaks per Cell	SD
14429	0.66	0.03
14743	0.08	0.03
14852	0.15	0.01
14744	0.07	0.01
14428	0.15	0.03
14431	1.15	0.02
14719	0.11	0.01
14726	0.08	0.02
16482	0.15	0.49
16483	0.14	0.00
16251	0.19	0.03
16254	0.62	0.02
16268	0.21	0.03
16959	0.32	0.00

Table II. a.) Mean breaks per cell in peripheral blood lymphocytes and corresponding EBV-immortalized cell line irradiated with 1 Gray. b) Descriptive statistics for peripheral blood lymphocytes and EBV-immortalized cell lines.

Patient ID	Blood MBC	EBV MBC
1	0.12	0.1
2	0.26	0.3
3	0.2	
4	0.16	0.2
5	0.32	0.4
6	0.1	
7	0.46	0.56
8	0.24	0.2
9	0.22	0.3
10	0.2	
11	0.19	0.2
12	0.17	0.16
13	0.32	0.32
14	0.33	0.5
15	0.62	0.47
16	0.24	0.28
17	0.2	0.18
18	0.22	0.31
19	0.54	0.44
20	0.2	0.2

Table III. Descriptive statistics for peripheral blood lymphocytes and EBV-immortalized cell lines.

	N	Minimum	Maximum	Mean	Std. Deviation
Peripheral Blood Lymphocytes	17	.12	.62	.2829	.13806
EBV-immortalized cell line	17	.10	.56	.3012	.13252

*Spearman correlation ρ= 0.907 and correlation is significant at 0.01 level.

Table IV. Descriptive statistics of MBC for affected (with cancer) and unaffected (without cancer) peripheral blood lymphocytes and EBV-immortalized cell lines.

	N	Minimum	Maximum	Mean	Std. Deviation
PBL_AFFECTED *	10	.16	.54	.2940	.12076
EBV_AFFECTED **	10	.20	.56	.3230	.11662
PBL_UNAFFECTED	7	.12	.62	.2671	.16869
EBV_UNAFFECTED	7	.10	.50	.2700	.15652

*Spearman correlation ρ=0.86 and correlation is significant at 0.01 level.
**Spearman correlation ρ=0.93 and correlation is significant at 0.01 level.

Figure Legends

Fig. 1. Mean breaks per cell of EBV-immortalized cell lines when a) assay done on different aliquots of same sample on the same day and b) assay done on different aliquots of same sample on different days.

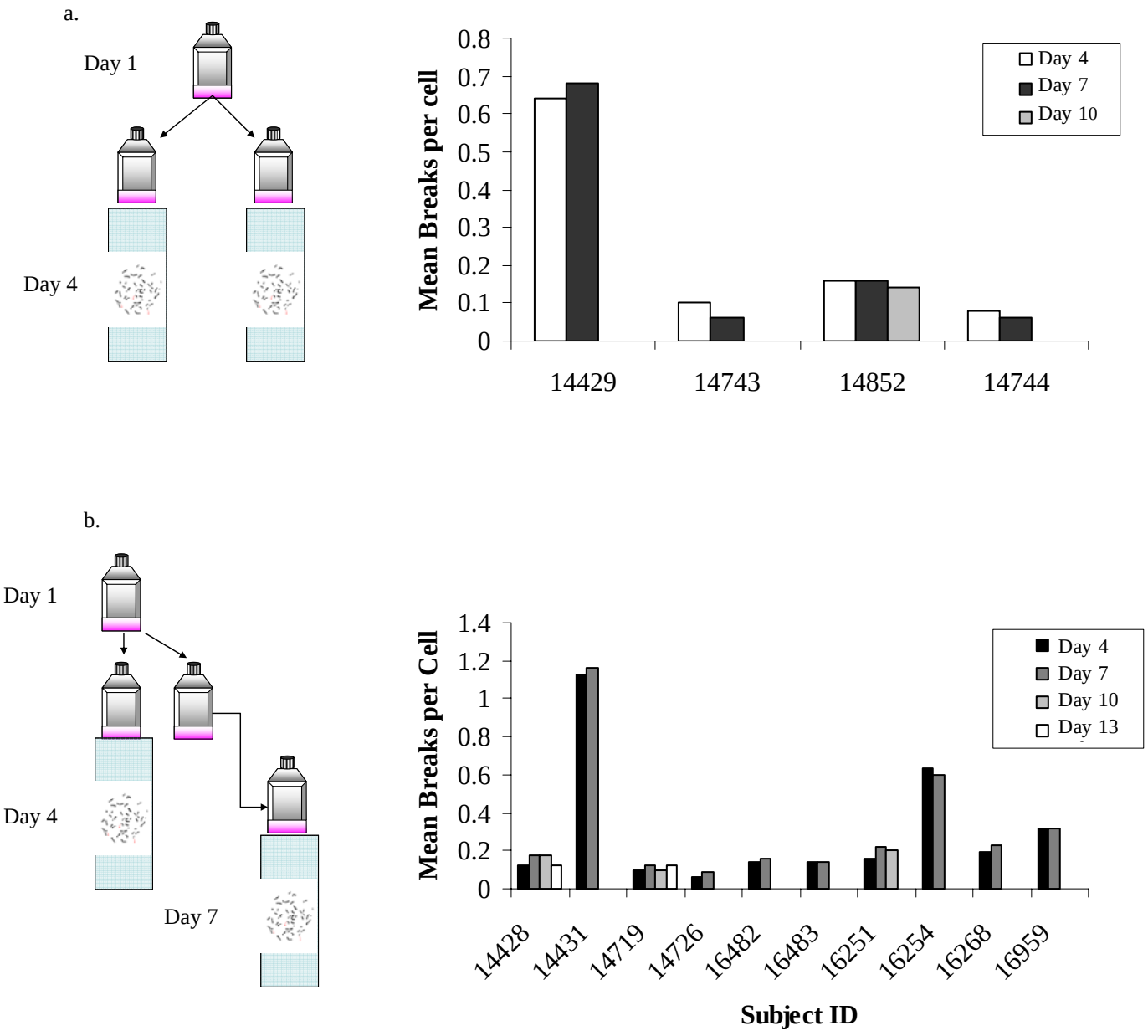


Fig. 2. Scatter plot of paired peripheral blood lymphocytes and EBV-immortalized cell lines. Solid line= best fit line; Dashed line is ρ=1

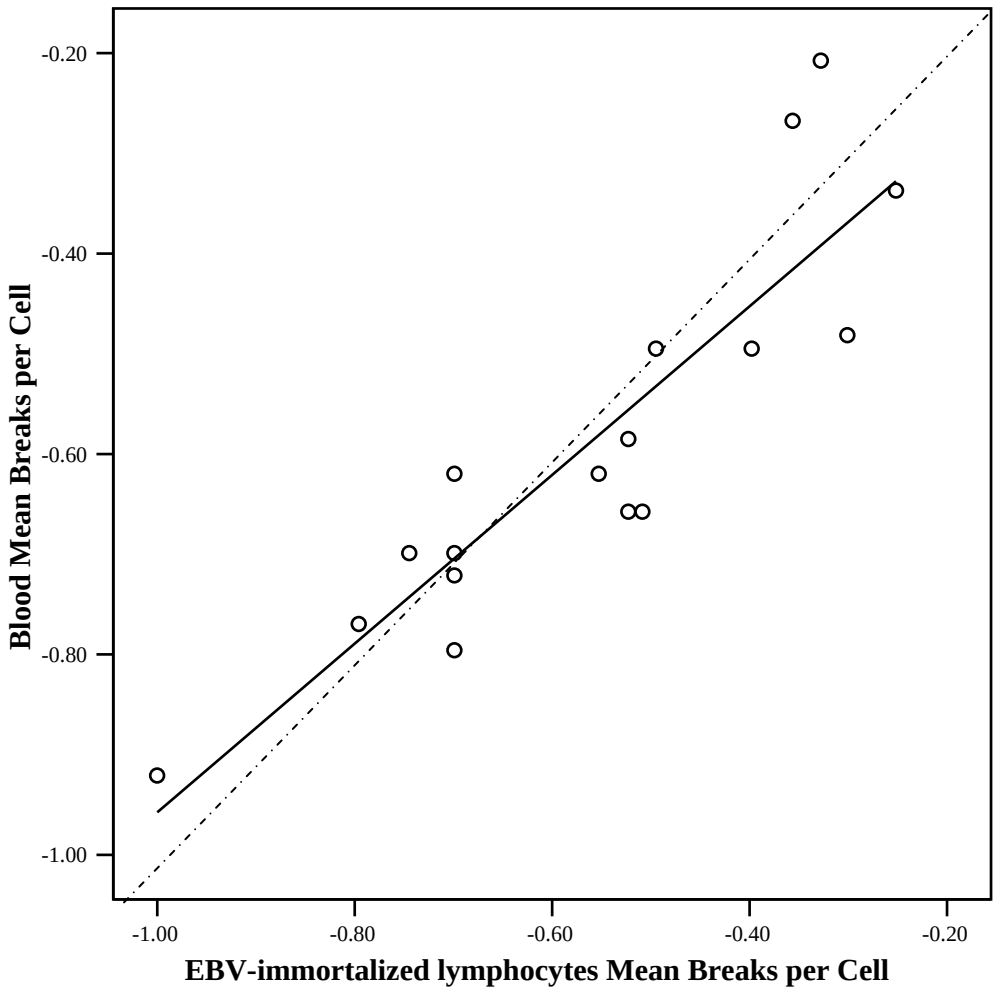
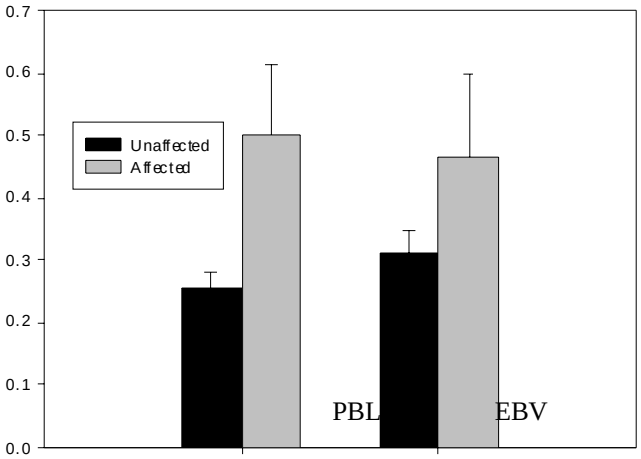
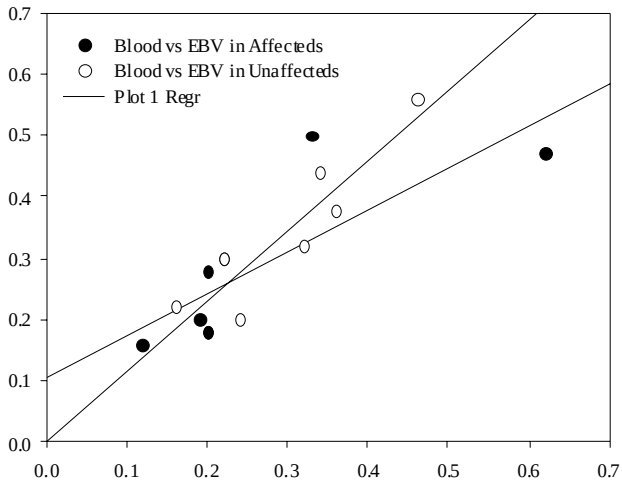


Fig. 3. Peripheral blood lymphocytes and EBV-immortalized cell line MBC (mean breaks per cell) stratified by cancer status. a) Bar graph shows that affected subjects will always have more mean breaks per cell. b) Spearman correlation for unaffected and affected is 0.92 and 0.86, respectively.

a



b



AACR 2006 Abstract

Induced chromosomal aberrations, genetics, and pathology in hereditary breast cancer

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Genetic breast cancer risk factors for both sporadic and familial breast cancer may be elucidated through studies of women with *BRCA1* mutations or in women in high risk breast/ovarian cancer families. There is variable penetrance for breast cancer among women in families with known BRCA1 mutations, and we hypothesize that this might be due to genetic variants in the wild-type allele of BRCA1 or other DNA repair genes. These genetic variants might also affect sporadic breast cancer risk. To identify genotype-phenotype relationships in DNA repair-related genes using EBV-immortalized cell strains from women in the Lombardi Cancer Center Familial Registry by assessing radiation-induced DNA repair capacity. Seventy-five affected and 57 unaffected *BRCA1* mutation carriers with immortalized cell lines were analyzed in this study. Their mean ages are 44 and 46, respectively ($p<0.01$). Gamma radiation-induced chromosomal breaks were measured as a marker of DNA repair capacity. The method was validated by assessing intra-individual variation in EBV-immortalized and peripheral blood lymphocyte test. Concordant samples were analyzed and spearman correlation was determined. *BRCA1* and *BRCA2* gene sequences were available and reviewed for the presence of genetic polymorphisms in relation to the number of chromosomal breaks using the chi squared test and unconditional logistic regression. To confirm that EBV-immortalized lymphocytes had little intra-individual variation, separate cultures were established and repeated measurements showed a high correlation ($r^2=0.994$), and a coefficient of variation of 12.37%. We assessed the relationship for the assay in freshly cultured lymphocytes and EBV-immortalized cell lines and found a high correlation ($r^2=0.865$, $p=0.01$). There was wide inter-individual variation for mean breaks per cell among the 130 women. Affected women had a greater number of mean breaks per cell than unaffected women (OR=3.2; CI: 1.5, 6.7). There was no association between *BRCA1* mutations (185/187delAG or 5382/5385insC), polymorphisms (E1038, K1183, L771L, P871L, S1613G, S694S) or haplotypes and DNA repair efficiency (n=32). There was also no association between *BRCA2* polymorphisms (H372N, K1132K, N289H) and DNA repair efficiency (n=32). Subjects with high mean breaks per cell were 2-fold (OR=1.8; CI: 0.9-3.6; $p=0.043$) more likely to be diagnosed with infiltrating ductal carcinoma and 4 times (OR=3.9; CI: 1.3-11.6; $p<0.01$) more likely to be diagnosed with metastatic breast cancer compared to subjects with low mean breaks per cell**.** The variable penetrance for breast cancer risk among BRCA1 carriers were not explained by common polymorphisms in BRCA1 or BRCA2, or haplotypes of BRCA1 in this model.